

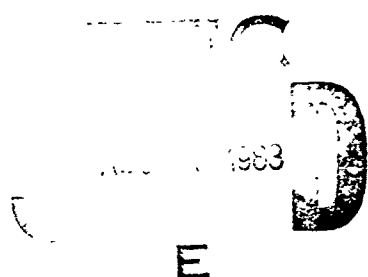
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INSTITUTE REPORT NO. 152

ACUTE INTRAMUSCULAR TOXICITY ( $LD_{50}$ ) OF  
1,1'-methylenebis [4-[(hydroxyimino) methyl] pyridinium] dibromide, (MMB-4)  
IN MALE MICE

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TOXICOLOGY GROUP,  
DIVISION OF RESEARCH SUPPORT



APRIL 1983

Toxicology Series 36

LETTERMAN ARMY INSTITUTE OF RESEARCH  
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

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In conducting the research described in this report, the investigation adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal Resources, National Research Council.

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(Signature and date)

7 April 1983

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)  The acute intramuscular toxicity of MMB-4 was determined in male ICR mice by using the single dose method. LD <sub>1</sub> , LD <sub>50</sub> , and LD <sub>95</sub> with their 95% confidence limits were calculated by probit analysis. The LD <sub>50</sub> was 448 mg/kg with the 95% confidence limit (398 mg/kg, 506 mg/kg). The MMB-4 formulation falls in the very toxic range. ✓		

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## ABSTRACT

The acute intramuscular toxicity of MMB-4 was determined in male ICR mice by using the single dose method. LD<sub>1</sub>, LD<sub>50</sub>, and LD<sub>95</sub> with their 95% confidence limits were calculated by probit analysis. The LD<sub>50</sub> was 448 mg/kg with the 95% confidence limit (398 mg/kg, 506 mg/kg). The MMB-4 formulation falls in the very toxic range.

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Justification	

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## PREFACE

TYPE REPORT: Acute Intramuscular Toxicity (LD<sub>50</sub>) GLP Study Report

TESTING FACILITY: US Army Medical Research and Development Command  
Letterman Army Institute of Research  
Presidio of San Francisco, CA 94129

SPONSOR: US Army Medical Research and Development Command  
Walter Reed Army Institute of Research  
Washington, DC 20012

PROJECT/WORK UNIT/APC: Medical Defense Against Chemical Agents,  
3~~8~~16277A875, WU 308, APC TL05  
5

GLP STUDY NUMBER: 82009

STUDY DIRECTOR: COL John T. Fruin, DVM, PhD, VC, Diplomate of  
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PRINCIPAL INVESTIGATOR: CPT Craig W. White, DVM, VC

CO-PRINCIPAL INVESTIGATOR: SP5 Leonard J. Sauers, MS

CONTRIBUTING INVESTIGATOR: CPT Martha A. Hanes, DVM, VC

PATHOLOGIST: LTC Paul W. Mellick, DVM, PhD, VC, Diplomate of  
American College of Veterinary Pathologists

STATISTICIAN: Virginia Gildengorin, PhD

REPORT AND DATA MANAGER: Carolyn M. Lewis, MS

REPORT AND DATA MANAGEMENT: A copy of the final report, study  
protocol, raw data, retired SOPs and  
an aliquot of the test compound will  
be retained in the LAIR Archives as  
GLP Study 82009.

TEST SUBSTANCES: 1,1'-Methylenebis[4-[(hydroxyimino) methyl]  
pyridinium] dibromide (MMB-4) (LAIR Code TW003)

INCLUSIVE STUDY DATES: 7 - 21 April 1982

OBJECTIVE: To determine the acute intramuscular toxicity potential of  
1,1'-Methylenebis [4-[(hydroxyimino) methyl] pyridinium]  
dibromide in male ICR mice.

#### ACKNOWLEDGMENTS

The authors wish to thank SSG Lance White, SP4 Larry Mullen, BS; SP4 Thomas Kellner, BS; and SP4 Evelyn Zimmerman for assistance in performing this research.

SIGNATURES OF PRINCIPAL SCIENTISTS AND MANAGERS INVOLVED IN THE STUDY:

We, the undersigned, believe the study number d2009 described in this report to be scientifically sound and the results in this report and interpretation to be valid. The study was conducted to comply, to the best of our ability, with the Good Laboratory Practice Regulations outlined by the Food and Drug Administration.

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REPLY TO  
ATTENTION OF

SGRD-ULZ-QA

14 February 1983

MEMORANDUM FOR RECORD

SUBJECT: Report of GLP Compliance

I hereby certify that in relation to LAIR GLP study 82009, the following inspections were made:

7 Apr 82  
15 Apr 82  
21 Apr 82

The report and raw data for this study were audited on 25 Oct 82.

Routine inspections with no adverse findings are reported quarterly, thus these inspections are also included in the July 1982 report to management and the Study Director.

A handwritten signature in black ink, reading "John C. Johnson", is positioned above the typed name.

JOHN C. JOHNSON  
CPT (P), MSC  
Quality Assurance Officer



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Acute Intramuscular Toxicity ( $LD_{50}$ ) of 1,1'-Methylenebis [4-[(Hydroxyimino) Methyl] Pyridinium] Dibromide, (MMB-4) in Male Mice--White, Sauers and Hanes

One of the goals of the Army Chemical Warfare Defense Program is to develop effective therapeutic compounds for the treatment of specific organophosphate intoxication. Compounds of low toxicity are sought for use since high peripheral concentration (related to effective dose) are necessary. The high peripheral concentration allows for the passage of quaternary ammonium oxime compounds through the blood-brain barrier to achieve therapeutic concentration at specific cholinesterase reactivation sites (1-3).

#### Objective of the Study

The objective of this study was to determine the acute intramuscular toxicity potential of 1,1'-methylenebis [4-[(hydroxyimino) methyl] pyridinium] dibromide in male ICR mice.

#### METHODS

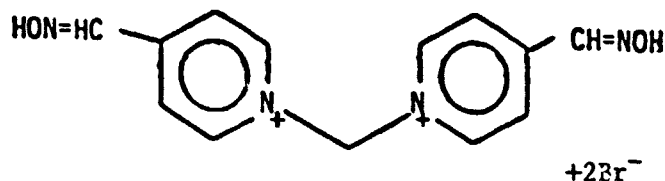
##### Test Substance

Chemical name: 1,1'-Methylenebis[4-[(hydroxyimino) methyl] pyridinium] dibromide, (MMB-4) (TA003)

Chemical Abstract Service Registry Number: None

Molecular formula:  $C_{13}H_{14}N_4O_2 \cdot 2Br$

Molecular structure:



Additional chemical data appear in Appendix A.

#### Animal Data

Animal data appear in Appendix B.

#### Environmental Conditions

Environmental conditions are described in Appendix C.

#### Dosing

A 25% weight/volume solution of MMB-4 in sterile water for injection (USP) was prepared between 0830-0845 on 7 April 1982.

Five dose levels (300 mg/kg, 400 mg/kg, 500 mg/kg, 600 mg/kg, and 700 mg/kg) were selected based upon the results of the Approximate Lethal Dose (ALD) Study which suggested a LD<sub>50</sub> of between 400 mg/kg and 500 mg/kg. Information obtained from US Army Institute of Chemical Defense indicated an IM LD<sub>50</sub> of 560 mg/kg (L. Harris, PhD, US Army Biomedical Laboratory, personal communication, 1982). The dose for each animal was calculated based on the animal's weight, the dose level desired, and the compound concentration in solution. The dose level was increased by graded volumetric increases rather than varying compound solution concentration. The intramuscular volume administered ranged from 40 ul (0.040 ml) to 107 ul (0.107 ml). Mice in the control group were untreated. A vehicle control group was not used due to the low toxicity of sterile water for injection (USP). The dosing material was injected into the caudal thigh muscle mass.

All animals were single dosed on 7 April 1982. Gas sterilized Hamilton gas-tight syringes (1705-LT) were used with sterile, disposable twenty-six gauge, one-half inch needles (Becton, Dickinson & Co.). The dosing procedures were conducted without animal sedation or anesthesia.

#### Observations

Animal observations were conducted and recorded daily commencing at 1400 hours. An additional observation period was conducted immediately following dosing. Animals were observed at 0700 hours on the day of sacrifice.

#### Statistical Methods

The LD<sub>1</sub>, LD<sub>50</sub>, LD<sub>95</sub> and slope determination were derived by Bliss probit analysis, as described by Finney (4).

Duration of Study

The study period was 14 days with a 7-day quarantine before the study onset.

Historical listing of study events appears in Appendix D.

Changes to Original Procedures

During dosing, Animal 154 (500 mg/kg dose group) did not receive the full calculated dose, and it was not included in the tabulation of the results.

## RESULTS

Mortality

Table 1 lists the compound-related deaths by group and the percent mortality.

TABLE 1

## Compound-Related Deaths by Group

Group	Dose Level	Compound-Related Deaths/	
		Number in Group	% Mortality
1	300 mg/kg	0/8	0%
2	400 mg/kg	2/8	25%
3	500 mg/kg	6/7*	86%
4	600 mg/kg	7/8	88%
5	700 mg/kg	8/8	100%
6	untreated controls	no mortality	

\*One animal eliminated because of misdosing.

Lethal Dose Calculations

Lethal dose (LD) values calculated by probit analysis for MMB-4 are given below for male ICR mice (Table 2). Figure 1 represents the dose response relationship curve when plotted as percent mortality versus dosage used. Figure 2 represents the dose response relationship curve with 95% confidence limits added.

TABLE 2\*

Lethal Dose (LD) Levels of MMB-4 in Male Mice

Percent Population	Lethal Dose (mg/kg)	95% Confidence Limits (mg/kg)
LD <sub>1</sub>	295	(207, 420)
LD <sub>16</sub>	375	(308, 456)
LD <sub>30</sub>	408	(351, 475)
LD <sub>50</sub>	448	(398, 506)
LD <sub>90</sub>	565	(468, 682)
LD <sub>95</sub>	603	(480, 757)

\*Statistician's report (Appendix E)

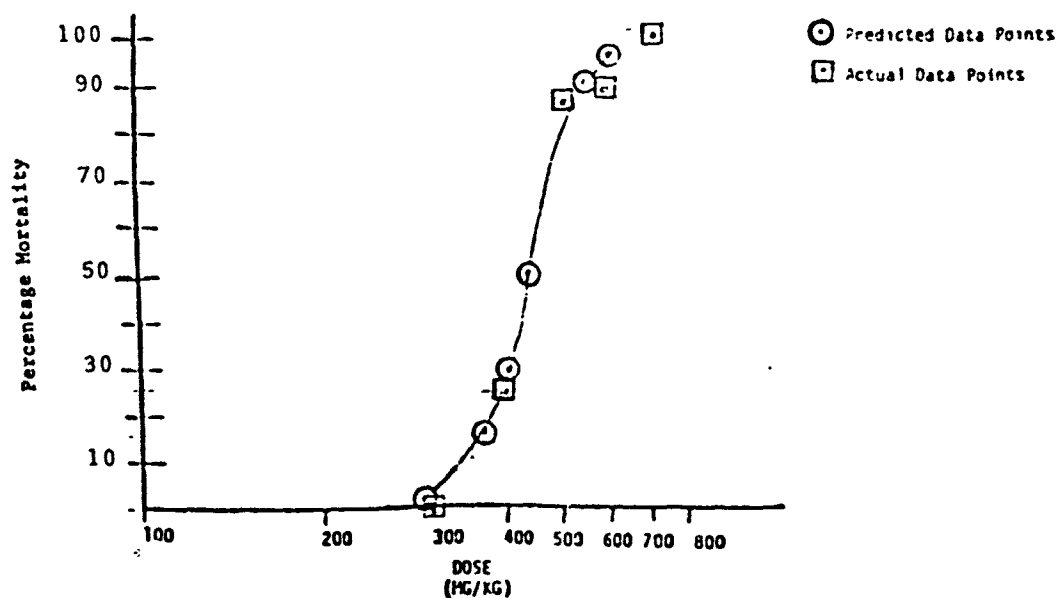


Figure 1: LD<sub>50</sub> for MMB-4, Probit Analysis Derived Dose Response Curve, Male ICR Mice.

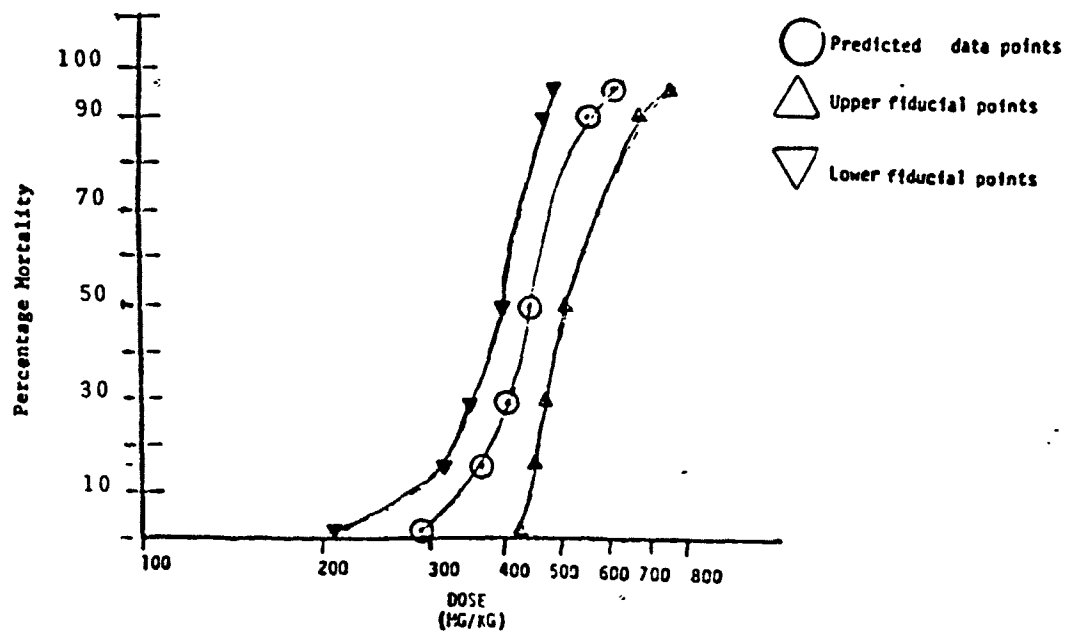


Figure 2: Lethal Dose for MMB-4, Probit Analysis Derived Response Confidence Limit Curve, Male ICR Mice.

### Clinical Observations

Animals were observed daily, subsequent to dosing, undisturbed in cages, outside of cages and after placement in cages. On the day of dosing, the animals were observed intermittently throughout the entire dosing procedure. Animals which exhibited clinical signs (other than slight depression) died quickly after the onset of signs. Typically, the animals developing clinical signs would at first become depressed and inactive. Development of muscle tremors and ataxia would then be accompanied by an increase in the respiratory rate. Breathing would become very shallow. Animals lost equilibrium and ability to ambulate. Cyanosis, which accompanied the onset of increased respiratory rate, was followed closely by agonal convulsions lasting 15 to 35 seconds.

Clinical signs indicated that the toxicity observed was of the anticholinergic-type. From clinical observation, it is reasonable to conclude that death occurred as a result of the neuromuscular blockade effect of MMB-4.

### Gross Pathological Observations

The veterinary pathologist's report appears in Appendix F.

### DISCUSSION

The calculated LD<sub>50</sub> for MMB-4 in male ICR mice was 448 mg/kg with a 95% confidence limit of (398 mg/kg, 506 mg/kg). The LD<sub>50</sub> is within the very toxic range (5).

Clinical signs of toxicity included increased respiratory rate, decreased respiratory depth, depression, inactivity, ataxia, loss of equilibrium and gait, cyanosis, tremors, convulsions and death. All animals (except 166) which developed clinical signs, other than mild depression, died during convulsions. Animal number 166 developed signs of intoxication but recovered to survive the observation period.

### CONCLUSION

The LD<sub>50</sub> for MMB-4 was determined to be 448 mg/kg in male ICR mice. The formulation is considered to be very toxic (5).

### RECOMMENDATION

MMB-4 should be considered for further safety testing for eventual human use, provided the efficacy is verified.



## REFERENCES

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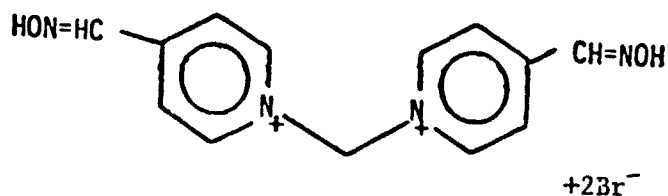
CHEMICAL DATA

1. Chemical name: 1,1'-Methylenebis[4-[(hydroxyimino)methyl]pyridinium]dibromide, (MIB-4)

Chemical Abstract Service Registry Number: None

Molecular formula:  $C_{13}H_{14}N_4O_2 \cdot 2Br$

Molecular structure:



Molecular weight: 418.10 g/mole

pH: N/A non-aqueous

Physical state: Fine yellow granular prisms

Decomposition temperature: 243-244 C

Stability: light - stable  
base - unstable  
heat - unstable  
non-hygroscopic

Contaminants: none known

Manufacturer: Ash Stevens Inc., Detroit Research Park  
5861 John C. Lodge Freeway  
Detroit, MI 48202

Published Toxicity Data: None known. Preliminary data  
furnished by the sponsor, indicates  
an IM LD<sub>50</sub> of 560 mg/kg in the  
male mouse.

2. Chemical name: Sterile water for injection, USP.

pH: 5.8

Contaminants: none known

manufacturer: Cutter Medical Division  
Cutter Laboratories Inc.  
Berkeley, California 94710

Manufacturer Lot Number: CH6255

ANIMAL DATA

Species: Mouse

Strain: ICR

Source: Hilltop Lab Animals  
P.O. Box 25  
Chetsworth, California 91311

Sex: Male

Age: 6 weeks at receipt

Method of Randomization: RANDOM Computer Program;  
SOP OP-ISG-21

Animals in each group: 8 male animals

Condition of animals at start of study: Normal

Body weight range at dosing: 31.5 - 38.8 g

Identification procedures: Tagging procedure (SOP-OP-ARG-1)  
modified to placement of tags  
in the skin over and in  
between shoulders using  
tag numbers between #101 and 171

Pretest conditioning: Quarantine 30 March - 6 April 1982

Justification: The laboratory mouse has been proven to be  
a sensitive and reliable system for  
lethal dose determination.

# ENVIRONMENTAL CONDITIONS

Caging: Number/cage: 5, except when mature males exhibited overly aggressive behavior, as few as 3 compatible animals were held per cage.

Type of cage used: Stainless steel wire top with a Polycarbonate "shoe box type" cage. Bedding = hardwood chips.

Diet: Certified Ralston Purina Rodent Chow Diet #5002 ad lib.

Water: Glass bottle, rubber stopper, lixit system.

Temperature:  $26 \pm 1$  C

Humidity:  $41 \pm 1\%$

Photoperiod: 0500-2000 hours/day (light, 15 hours)

HISTORICAL LISTING OF STUDY EVENTS

30 Mar 82	50 male ICR mice were received at LAIR. Mice were housed in groups of 3 to 5 and tagged on the skin between and above the shoulder. Animals were weighed and 2 animals were submitted for quality control necropsy.
2 Apr 82	Animals were randomized, weighed, dosed and observed immediately. Animals that died were submitted for gross necropsy.
8 - 21	All animals were observed daily for clinical signs.
9,12,15,19,21 Apr 82	All animals weighed.
21 Apr 82	All surviving animals were weighed, sacrificed and gross necropsied.

## Statistical Analysis

Eight male animals were assigned to each of six dose groups by simple random sampling techniques using a program, Random, on the Data General C 330 computer.

Miss method of probit analysis was used to determine the  $LD_1$ ,  $LD_{50}$  and  $LD_{95}$  values along with the corresponding 95% confidence limits (Table 2). The program, PROBIT, was used to determine the probit curve and the lethal dose values. The probit regression line fit to the data was

$$Y = -28.39 + 12.78 \log X,$$

where X is the dose and Y the corresponding probit value.

*Virginia L. Gildengorin*

VIRGINIA L. GILDENGORIN, PhD

DAC

Statistician

22 September 1982



Gross Pathology Summary and Interpretation of GLP Study  
62-009; intramuscular LD<sub>50</sub> 113-4, male ICR ice

The deaths of 2/3\* male mice in Group 2 (400 mg/kg), 6/7 mice in Group 3 (500 mg/kg), 7/8 mice in Group 4 (600 mg/kg) and 8/8 mice in Group 5 (700 mg/kg) were attributed to the toxic effect of the tested compound. All of the deaths occurred between 4 minutes and 25 minutes following administration of the test compound by intramuscular injection. The average time from injection of the test compound to death by dosage group was: 20 minutes for Group 2, 8.6 minutes for Group 3, 6.7 minutes for Group 4, and 5.7 minutes for Group 5. None of the mice in Group 1 (300 mg/kg) or Group 6 (control) died prior to the scheduled termination of the study 14 days after injection of the test compound. One mouse was removed from Group 3 because of misdosing and data from that animal will not be included in this report.

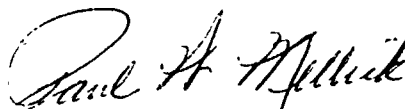
Gross lesions attributable to the test compound were present in all of the groups in which deaths occurred but not in every animal. These lesions consisted of hemorrhage and/or congestion in the lung which was observed in the following dosage group frequency: 2/8 in Group 2, 5/7 in Group 3, 2/8 in Group 4, and 1/8 in Group 5. Gross hemorrhage was observed at the injection site in 3/8\* in Group 2, 6/7 in Group 3, 6/8 in Group 4, and 8/8 in Group 5. With the exception of one animal in Group 2, hemorrhage at the injection site was only observed in animals that died. It is likely that evidence of hemorrhage in animals surviving for the 14 day observation period would have been cleared during that time.

Other lesions observed in mice on this study included corneal opacity in 1/7 mice in Group 3, hypopyon in 1/8 mice in Group 4, lens opacity in 1/8 in Group 5, bite wounds in 1/7 from Group 3 and 1/8 in Groups 4, 5, and 6, hydrophrosis in 2/8 mice in Group 4, yellow spots on kidneys of 1/8 mice in Group 4 and distal end of tail missing in 2/8 mice in Group 5. These changes were considered to be incidental lesions that were unrelated to administration of the test compound.

In summary, the average time between intramuscular injection of the test compound and death in mice decreased with increased dosages of the material. The gross pathologic effects in addition to death that were most likely due to a single intramuscular injection of 14B-4 that were observed in male ICR mice in this study were congestion and/or hemorrhage in the lung. Hemorrhage at the injection site was commonly observed but was more likely the result of trauma than an effect of the test material.

\*Number of mice affected/Number of mice in the group.

Necropsies revealed no test compound-related lesions in male ICR mice that were killed at the termination of the study.



PAUL W. MELLICK, DVM, PhD  
Diplomate, ACVP  
LTC, VC  
Chief, Pathology Services Group  
Division of Research Support

30 July 1982

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